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Carlsen, Thomas Gelsing

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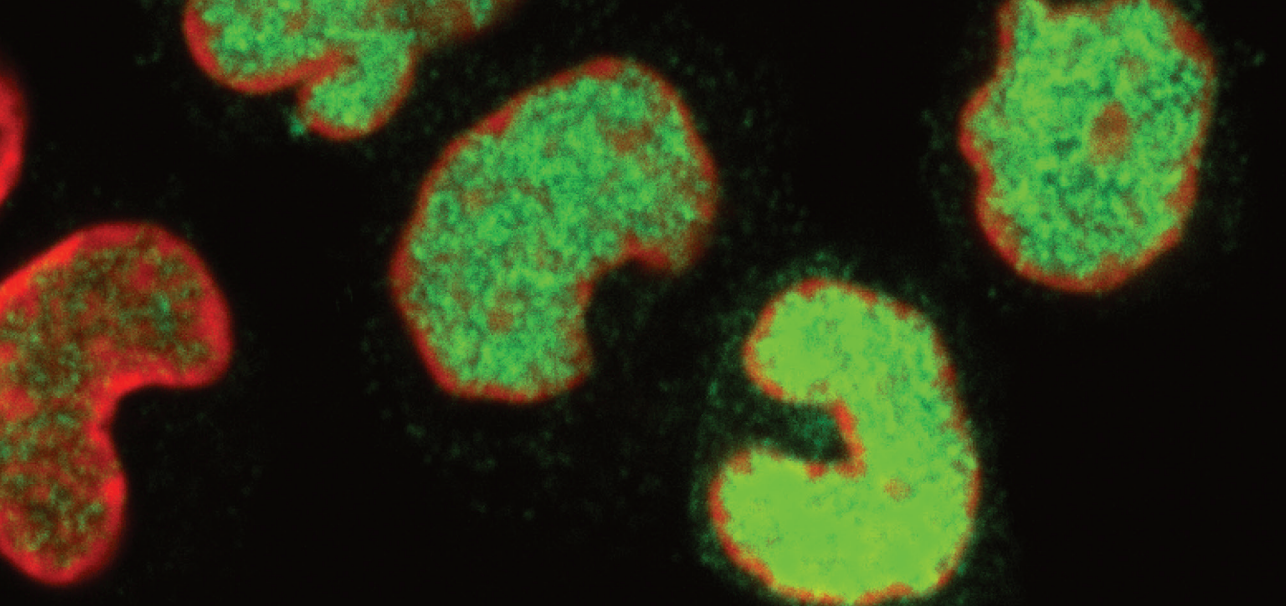

MOLECULAR PATHOGENESIS OF SPONDYLOARTHRITIS

INVESTIGATING THE CELLULAR ALARMINS HEAT SHOCK PROTEIN 60 AND INTERLEUKIN-1 ALPHA

BY
THOMAS GELSING CARLSEN

DISSERTATION SUBMITTED 2015

AALBORG UNIVERSITY
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Thomas Gelsing Carlsen

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PhD supervisor:	Professor Svend Birkelund Aalborg University
PhD committee:	Associate Professor Cristian Pablo Pennisi (chairman)
	Professor Steffen Thiel
	Assigned Professor Mirja H. Puolakkainen
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PREFACE

After 3 years of hard work my PhD life is coming to an end. It has been a journey of intense research giving birth to a lot of challenges but certainly also professional and personal development.

The groundwork for this PhD thesis was carried out at Aarhus University in 2011 where I obtained a master degree in Biology. Looking back at my first encounter with a medical laboratory at the Department of Biomedicine in Aarhus I must admit that the last 4 years have changed the way that I understand and think biological science. Moreover, accepting that the title biologist does not mean "plants and animal expert" but simply means having an interest in exploring and explaining the functioning of living organisms. In my case this has so far been mostly related to human biology and the investigation of immunological dysfunction. The research experience that follows a PhD programme has given me a comprehensive knowledge of the scientific process from hypothesis to methodology, laboratory work, data analysis and presentation, which will make it easier to engage in scientific problem solving in the near future. More so, accepting mistakes as a part of this process. The ability to combine different research tools to create data suitable for publication has been very satisfying and I no longer have any problems with the title researcher, because I actually believe it suites me well.

This Dissertation is constructed with an opening introduction followed by a brief description of the methodological approaches and concluded in a summarizing discussion. The aim of the thesis is presented through research hypotheses, which are further discussed in 4 scientific papers of which one of them (paper III) is a review that discuss findings in paper I & II.

I would like to thank my parents and brother, Lis Carlsen, Lars Gelsing and Mikael Gelsing Carlsen for moral support. In addition, I would like to thank everyone involved in my research especially laboratory technicians Karin Skovgaard Sørensen and Ditte Bech Kristensen. A special thank to Supervisors Svend Birkelund and Gunna Christiansen. I would also like to thank the Amnon group at the La Jolla institute for Allergy and Immunology in San Diego for being friendly and open, making my three months stay as visiting researcher a worthwhile experience.

Thomas Gelsing Carlsen

ABSTRACT

The Spondyloarthritis (SpA) comprise a heterogenic group of 5 diseases in which immunological dysfunction give rise to a number of shared symptoms used for diagnosis. Most notably, these include sacroilitis, spondylitis and the presence of enthesitis. Treatment is a combination of anti-inflammatory drugs and exercise to relieve the patient from pain and risk of spinal fusion. However, as no cure is at hand ongoing research seeks to improve the understanding of the underlying mechanism responsible for SpA pathogenesis, which is also the overall objective of this thesis. Several hypotheses are retrieved from the literature, which since the discovery of the susceptibility gene *HLA-B27* have moved from interest in the antigenic presentation of peptides to CD8+ T cells and autoimmune reactions born from protein homology to exploring the presence of single nucleotide polymorphisms (SNP) in the genotype of SpA through genome-wide association studies. The polygenic nature of these diseases is a challenge, as the genetic background of SpA seems to be significant only in combination with environmental stimuli such as bacterial infections.

To target this complexity the following hypotheses were constructed 1) Anti-HSP60 antibodies are linked to disease activity in SpA 2) IgG subclass determination of the Anti-HSP60 response in SpA does not support cross-reactivity 3) SNP's in the *IL-1A* gene affect kinetics in the IL-1α response. These hypotheses have generated 3 scientific research papers which content is outlined below.

The first study was conducted on 82 serum samples collected from patients with symptoms restricted to the axial skeleton. The objective for this study was to investigate if a relationship between antibodies to the immunogenic heat shock protein 60 (HSP60) and clinical characteristics exist. Recombinant HSP60 was purified for human and 3 different bacteria known to trigger SpA disease. Furthermore, an enzyme-linked immunosorbent assay (ELISA) was established to determine levels of anti-HSP60 IgG subclass antibodies. The amino acid similarity between bacterial and human HSP60, also named molecular mimicry, has generated belief that cross-reactive antibodies exist with possible implication in SpA pathogenesis. However, our results demonstrated no significant higher levels of antibodies to bacterial HSP60 between SpA patients and healthy controls. Moreover, while antibodies against human HSP60 were associated with SpA and

predominantly of the IgG3 subclass, antibodies against bacterial HSP60 are predominantly of the IgG1 subclass. Therefore, based on the ELISA results, cross-reaction between bacterial and human HSP60 could not be supported as a pathogenic mechanism for SpA disease in our study. However, a weak correlation between anti-human HSP60 IgG3 and one of the several disease parameters was demonstrated, which generated interest in conducting a follow-up study in which the same patients were enrolled and again asked for blood donation.

The second study was a cohort study in which two serum samples from the same patient collected with a 4-year interval, were analyzed. Antibodies measured in the first study could now be determined over time. Hence, this study investigates the association between antibody levels and disease progression. Unfortunately only 39 patients (48%) of the samples already analysed in paper I was collected. Probably due to the reduced number of patients participating, no correlation between disease parameters and antibodies was demonstrated. Again, the IgG subclass specificity for bacterial and human HSP60 was presented with a dominance of IgG3 for human HSP60 and IgG1 for bacterial HSP60 rejecting the hypothesis of cross-reactivity.

The third study was based on the fact that polymorphisms in genes involved in innate immune recognition and cytokine signaling pathways have been demonstrated among SpA patients. Among these genes is *IL-1A*, which is considered a pro-inflammatory cytokine produced mainly by macrophages at the site of infection. IL-1α is synthesized as a 33 kDa precursor peptide that in mice is cleaved by a calpain-like protease to a nuclear-associated 16 kDa propiece and a secreted 17 kDa mature IL-1α peptide. It is apparent from the literature that a functional assay to validate the importance of SNPs in the IL-1α gene is needed. Therefore, we made a time-course study in which human machrophages enriched from peripheral blood was stimulated with a bacterial endotoxin named lipopolysaccharide (LPS) and stained for the propiece with a monoclonal antibody (MAb) generated against recombinant precursor IL-1α. Moreover, by conjugating the MAb to a fluorophore (FITC) we conducted a flow cytometric analysis to investigate the IL-1α kinetics among human monocytes. With the use of confocal microscopy IL-1α propiece was demonstrated to localize to the nucleus already 2 hrs post LPS stimulation with increased intensity at 12 and 20 hrs post LPS stimulation. Interestingly, a western blot analysis of LPS-stimulated human monocytes could not detect the propiece cleavage product. Finally, CD68 proved to be a valid marker for

IL-1α producing monocytes. The kinetics of IL-1α protein production followed a saturation curve profile while mRNA production was strongest at 4 hrs.

In conclusion, the findings of this thesis have demonstrated **1)** That antibodies against bacterial and human HSP60 are not markers for SpA disease progression. **2)** That IgG subclass specificity exist for human (IgG3) and bacterial (IgG1) HSP60 not supporting cross-reactivity as a pathogenic trigger in SpA disease. **3)** That IL-1α propiece localize to the nucleus of human macrophages and increases exponentially over time up until 20 hrs post LPS stimulation. Based on the findings of this thesis the long-lived hypothesis of molecular mimicry, as a pathogenic trigger in SpA pathogenesis seems unlikely. Moreover, a path is set for a clinical study in which donors sequenced for the *IL-1A* gene is validated with measurements on IL-1α kinetics from the method developed here. This could provide important knowledge on a potential functional role for *IL-1A* SNP's in SpA pathogenesis.

DANSK RESUMÉ

Spondylartrit (SpA) omfatter en heterogen gruppe af 5 sygdomme, hvor immunologisk dysfunktion giver anledning til en række fælles symptomer anvendt til diagnosticering. Mest bemærkelsesværdigt omfatter disse sacroilitis, spondylitis og enthesitis. Behandling er en kombination af anti-inflammatoriske lægemidler og øvelser der aflaster patienten fra smerte og risiko for spinal fusion. Da der ikke findes en kur for SpA forsøger igangværende forskning imidlertid at forbedre forståelsen af den underliggende mekanisme der er ansvarlig for udviklingen af sygdommen. Dette er også det overordnede formål med denne afhandling. Adskillige hypoteser kan hentes fra litteraturen, som siden opdagelsen af susceptibilitetsgenet *HLA-B27* er flyttet fra interesse i den antigene præsentation af peptider til CD8+ T-celler og autoimmune reaktioner affødt af protein homologi til at udforske tilstedeværelsen af enkelt nukleotid-polymorfier (SNP) i genotypen ved SpA patienter gennem genom-dækkende associationsstudier. Den polygeniske karakter af disse sygdomme er en udfordring, idet den genetiske baggrund for sygdom synes at være væsentlig kun i kombination med miljømæssige stimuli, såsom bakterielle infektioner.

For at omfatte denne kompleksitet blev følgende hypoteser konstrueret 1) anti-HSP60 antistoffer er forbundet med sygdomsaktivitet i SpA 2) IgG subklasse bestemmelse af anti-HSP60 responset støtter ikke hypotesen om krydsreaktivit ved SpA 3) SNP'er i *IL-1A* genet har indflydelse på kinetikken i IL-1α responset. Disse hypoteser har genereret 3 videnskabelige forskningsartikler, hvis indhold er skitseret nedenfor.

Det første studie var en serologisk undersøgelse af 82 serumprøver fra patienter med symptomer begrænset til rygsøjlen. Målet for dette studie var at undersøge, om der eksisterer en sammenhæng mellem antistoffer mod det immunogene heat shock protein 60 (HSP60) og kliniske karakteristika for SpA diagnose. Rekombinant HSP60 blev oprenset for human og 3 forskellige bakterier kendt for at udløse SpA sygdom. Endvidere blev et enzymatisk assay (ELISA) etableret for at bestemme niveauer af anti-HSP60 IgG subklasse antistoffer. Aminosyre lighed mellem bakteriel og human HSP60, også kaldet "molecular mimicry", har genereret tro på, at kryds-reaktive antistoffer kan være involveret i SpA patogenesen. Men vores resultater viste ingen signifikant højere niveauer af antistoffer til bakteriel HSP60

mellem SpA patienter og raske kontroller. Desuden, var antistoffer mod human HSP60, forbundet med SpA, overvejende af IgG3-underklassen, hvorimod antistoffer mod bakteriel HSP60 var overvejende af IgG1-underklassen. Derfor kunne kryds-reaktivitet mellem human og bakteriel HSP60 ikke støttes som en patogen mekanisme for SpA sygdom. Desuden blev en svag korrelation mellem anti-humant HSP60 IgG3 og en af de mange sygdomsparametre demonstreret, hvilket genererede interesse for at gennemføre en opfølgende undersøgelse, hvor de samme patienter blev indkaldt igen.

Det andet studie var et kohorte studie med 2 serumprøver fra samme patient med et 4 års interval. Antistofferne målt i dette studie kunne da bestemmes over tid. Der er altså her tale om en undersøgelse af sammenhæng mellem antistof niveau og sygdomsforløb. Desværre blev kun 39 patienter (48%) af prøverne der var blevet analyseret i paper I indsamlet. Sandsynligvis på grund af det reducerede antal af patienter der deltog, kunnne der ikke påvises nogen sammenhæng mellem sygdomsparametre og antistoffer. Igen blev IgG subklasse specificitet for bakterielt og humant HSP60 præsenteret med en dominans af IgG3 til humant HSP60 og IgG1 for bakterielt HSP60 hvormed hypotesen om krydsreaktivitet igen kunne afvises.

I den tredje undersøgelse udforskes det faktum, at polymorfier i gener involveret i den medfødte immungenkendelse og cytokin-signalveje er blevet påvist blandt SpA patienter. Blandt disse gener er *IL-1A*, der betragtes som en pro-inflammatorisk cytokin produceret primært af makrofager på stedet for infektion. IL-1α syntetiseres som et 33 kDa forstadium peptid, som spaltes i mus af en calpain-lignende protease til et nukleært-associeret 16 kDa propiece og et udskilt 17 kDa modent IL-1α peptid. Da behovet for et funktionelt assay til at validere betydningen af SNP'er i IL-1α-genet fremgår af litteraturen lavede vi et tidsforsøg, hvor humane makrofager beriget fra perifert blod blev stimuleret med et bakterielt endotoxin navngivet lipopolysaccharid (LPS) og farvet for propiece med et monoklonalt antistof (MAb 1.3.2), der var genereret mod rekombinant precursor IL-1α. Endvidere ved at konjugere MAb1.3.2 til et fluorofor (FITC) lavede vi en flow cytometrisk analyse af IL-1α kinetikken hos humane monocytter. Med anvendelse af konfokal mikroskopi blev IL-1α propiece påvist at lokalisere til kernen allerede 2 timer efter LPS-stimulering med øget intensitet på 12 og 20 timer efter LPS-stimulering. Desuden viste CD68 sig at være en gyldig markør for IL-1α producererende monocytter. Kinetikken for produktionen af IL-1α protein fulgte en mætningskurve, mens mRNA produktion var stærkest ved 4 timer.

Sammenfattende har resultaterne af denne afhandling vist **1)** At antistoffer mod bakterielt og humant HSP60 ikke er en markør for SpA sygdomsaktivitet. **2)** At IgG subklasse specificitet findes for human (IgG3) og bakteriel (IgG1) HSP60 og ikke understøtter krydsreaktivitet som en patogen udløser i SpA sygdom. **3)** At IL-1α propiece lokalisere til kernen af humane makrofager og stiger eksponentielt over tid indtil 20 timer efter LPS-stimulering. Baseret på resultaterne af denne afhandling er den aldrende hypotese om molekylær mimicry, som en patogen udløser i SpA patogenese, usandsynlig. Dertil er der udviklet en platform tilgængelig for en klinisk undersøgelse, hvor donorer sekventeret for *IL-1A* gen variation valideres med målinger på IL-1α kinetik fra den udviklede metode i paper IV. Dette kan give vigtig viden om en potentiel funktionel rolle for *IL-1A* SNP'er ved SpA patogenesen.

ABBREVIATIONS

ADCC Antibody-Dependent Cell-mediated Cytotoxicity

ASAS Assessment of Spondylo-Arthritis international Society

ATP Adenosine TriPhosphate

CD Cluster of Differentiation

ESSG European Spondylarthropathy Study Group

FSC Forward Scatter

FITC Fluorescein IsoThioCyanate

GWS Genome-Wide association Studies

Hc Heavy chain

HGNC HUGO Gene Nomenclature Committee

HLA Human Leukocyte Antigen

HSP Heat Shock Protein

Ig Immunoglobulin

IκB Inhibitor of NFκB

IL InterLeukin

ITAM Immunoreceptor Tyrosin-based Activation Motif

Lc Light chain

MyD Myeloid Differentiation

MHC Major Histocompatibility Complex

MRI Magnetic Resonance Imaging

NCBI National Center for Biotechnology Information

NFκB Nuclear Factor kappa-light-chain-enhancer of activated B cells

NGS Next Gene Sequencing

NLR NOD-Like Receptor

PAMP Pathogen-Associated Molecular Patterns

PBMC Peripheral Blood Mononuclear Cells

PMT Photo Multiplier Tube

RAcP Receptor Accessory Protein

SpA SpondyloArthritis

SNP Single Nucleotide Polymorphism

SSC Side Scatter

TCR T Cell Receptor

TIR Toll-Interleukin Receptor

TIRAP Toll-Interleukin 1 Receptor domain containing Adaptor Protein

TLR Toll-Like Receptor

TNF Tumor Necrosis Factor

TABLE OF CONTENTS

SCIENTIFIC CONTRIBUTION

Paper I - Increased levels of IgG antibodies against human HSP60 in patients with spondyloarthritis, PloS One, Vol. 8, 2013.

Paper II - IgG subclass antibodies to human and bacterial HSP60 are not associated with disease activity and progression over time in axial spondyloarthritis, Arthritis Research and Therapy, Vol. 15, 2013.

Paper III - A role for anti-HSP60 antibodies in arthritis: a critical review, OA Arthritis, Vol. 1, 2013.

Paper IV - Interleukin-1 alpha activation and localization in lipopolysaccharide-stimulated human monocytes and macrophages, Journal of Immunological Methods, Vol. 422, 2015.

Not part of thesis - Neutrophil extracellular traps in ulcerative colitits: a proteome analysis of intestinal biopsies, Inflammatory Bowel Diseases, Vol.21, 2015.

1. INTRODUCTION

1.1 SPONDYLOARTHRITIS

Spondyloarthritis (SpA) constitute a group of 5 immune-mediated inflammatory diseases: Ankylosing spondylitis (AS), Inflammatory bowel disease (IBD) arthritis, Reactive arthritis (ReA), Psoriatic arthritis (PsA) and undifferentiated spondyloarthritis (uSpA) (figure 1A). The name SpA comes from the two Latin words spondyl and arthropathy meaning vertebra and unspecific joint disease, respectively. Together the two words accentuate the most prominent clinical features shared among the various diseases affecting the spine (spondylitis), joints (arthritis), ligament and tendons (enthesitis) (figure 1B).

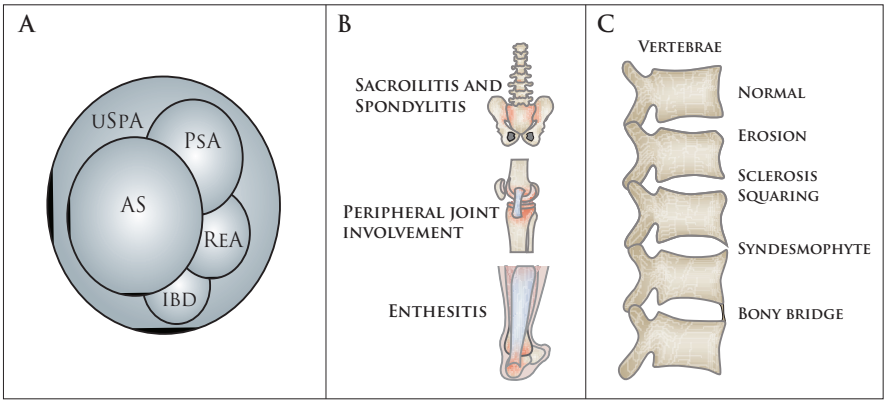


Figure 1: A) A venn diagram illustrating the different disease groups of SpA. **B)** The shared symptoms of SpA (modified from Rosenbaum *et al.* 2012 [1]) **C)** The consequences of inflammation and ossification on the spinal vetrebraes in AS (modified from Maksymowych 2010 [2]).

The most promintent pathological feature seen among SpA patients is inflammation. Under normal conditions this process is activated because foreign material is recognized by the immune system. This activates specific immune cells and triggers them to release toxic substances to combat the intruider. However, in SpA diseases this process is out of control as it for some unknown reason attacks the synovium of the sacroiliac- and spinal joints. The resulting inflammation is continuosly damaging the tissue, which is being counteracted by a reperative response that induce pathological bone calcification. This in turn causes the joints to loose their mobility. By means of radiography this is visualized by fusion of the vertebrae accompanied

with the development of so-called syndesmophytes (figure 1C). Initially, changes occur in the lumbar spine but will gradually spread upwards. Squaring of vertebral bodies is the first indication, which is followed by erosions and sclerosis of the anterior corners of the vertebral bodies and ossification of the annulus fibrosus (syndesmophyte formation). This pathology is characteristic for patients suffering from AS, which is considered to be the prototype of SpA. If not treated the calcification of spinal joints in these patients give rise to a characteristic curved posture also refered to as the "bamboo spine".

It is estimated that SpA as a group occurs in 0.5 to 1% of the population in Europe. Incidence rate of AS has been reported to be 7.3 per 100,000 adults in Norway and 6.9 per 100,000 in Finland. It affects people of all age groups, but with a preference for men (2-3:1) at the age of 20-30 [3].

Globally, 90% of AS patients are *HLA-B27* positive. The *HLA-B27* allele is located on chromosome 6 and codes for a major histocompatibility complex I (MHC-I) variant. MHC molecules are classed into MHC-I and MHC-II based on function and which cells they are present in. MHC-I molecules are constantly being processed and transported to the surface of all nucleated cells in the body. Here they present intracellular (cytosol) peptides (8-11 amino acids) to the adaptive immune system (figure 2A) through cytotoxic $CD8^+$ T cells. If the $CD8^+$ T cell recognizes the peptide as foreign (e.g. viral) the cell is killed. This creates a surveillance system in which the diversity of MHC class molecules and their peptide specificity are essential to manage the magnitude of different pathogens with preference for the human cell.

Several hypotheses have been suggested explaining the association of SpA to *HLA-B27* [5]. One such hypothesis links HLA-B27 misfolding to the interleukin-23 (IL-23) production through an unfolded protein response. As IL-23 is involved in the differentiation of CD4+ T cells into the proinflammatory Th17 lymphocytes this hypothesis has been supported by the high plasma IL-17 levels detected in patients with AS [6]. An older hypothesis, disussed in the next section, proposes that bacterial infection is the trigger of arthritis. A strong support for the involvement of bacterial infection in SpA is ReA (figure 1A), a non-purulent joint inflammation that usually follows a bacterial gastrointestinal or urogenital infection with a time interval of 1-3 weeks.

The association of AS to *HLA-B27* is strong. However, only 5% of *HLA-B27* positive individuals in the population develop SpA. This suggests the impact of other factors in the development of SpA and focus in the litterature has recently turned to the innate immune response to discover new contexts (figure 2B) [6].

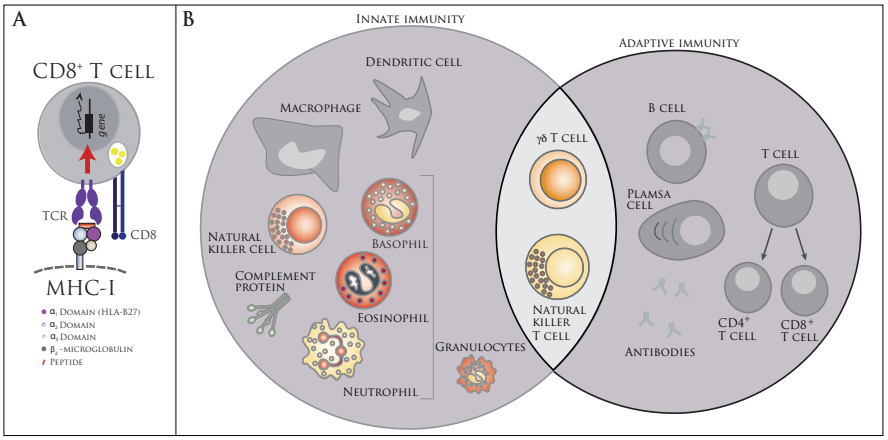


Figure 2: A) The interaction between a $CD8^+$ T cell and the major histocompatibility complex I (MHC-I) in which the *HLA-B27* allele encodes the α_1 domain involved in peptide recognition. **B)** The response of the human immune system can be divided into two parts described in the literature as innate and adaptive highlighting the difference in its development and ability to neutralize and remove pathogens from the human body. The initial mechanism of defence is performed by the innate immune response. It includes the skin and a variety of cells and soluble factors presented in the figure above (modified from Dranoff, 2004). The recognition mechanisms of these cells is encoded in their genetic materiel and therefore termed innate. However, on the other hand, the adaptive immune response takes time to mature as it selectively improves its affinity for the pathogen and built up memory. It is presented by the following components: antibodies, B cells and T cell, which are divided into CD4+ and CD8+. Other cells with cytotoxic behaviour named γδ T cells and natural killer cells are believed to be involved with both innate and adaptive responses [7].

Treatment of patients is mainly carried out with an expensive anti-tumor necrosis factor (TNF) and nonsteroidal anti-inflammatory drug therapy, initiated to stop disease activity and development. After the acute phase of disease has ended patients are usually referred to a physiotherapist to begin training of joints to strengthen muscles and ligaments. Disease activity and progress is monitored by the use of blood tests, X-ray pictures, magnetic resonance imaging (MRI) scans and different indexes: Bath Ankylosing Spondylitis Functional, Disease and Metrology index (BASFI, BASDI and BASMI) [8]. Even though there is no cure the outcome of disease is very much dependent on early diagnosis for prevention of severe musculoskeletal symptoms.

For diagnosis, the rheumatologist combines the blodtest for HLA-B27 analysis with patient anamnesis, a physical examination of the back and hips and finally MR-scans to detect bilateral sacroilitis and inflammatory back pain. Because the diverse clinical presentations seen among SpA patients may have a common background a number of different sets of criteria have been developed as to facilitate the diagnosis. These include: Amor, ESSG, ASAS and the (modified) New York criteria, which are reviewed elsewhere [3].

1.1.1. CROSS-REACTIVE ANTIBODIES TO HSP60

In the 1980s a group of scientists made a rat model of juvenile arthritis (JRA) in which they identified a T cell specific for a sequence (epitope) within the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) antigen. Administration of this antigen to rats induced resistance to subsequent attempts to produce JRA and this molecule was therefore suggested to play a role in immune-regulation [9]. The antigen was later identified as the 65-kD mycobacterial heat-shock protein (HSP) or GroEL, which belongs to the HSP60 family of bacterial heat shock proteins [10]. Proteins within this family have shown to be highly conserved throughout evolution with close to 50% amino acid identity to its mammalian counterpart [11]. These findings led immunologists to believe that self-antigens, in this case HSP60, might be able to break immune tolerance as a result of "molecular mimicry" as outlined in figure 3 [12]. The history of this theory is reviewed in more detail in paper III [13]. Immune tolerance is a concept that explains the different mechanisms including central and peripheral tolerance that inactivates the immune response upon recognition of unharmful substances.

The human HSP60 is an endogenous protein located within the mitochondria and today known to be involved in the transportation and refolding of proteins from the cytoplasm into the mitochondrial matrix. Because these proteins are involved in protein guidance, they have been given the name chaperones. Several HSP families exist and are designated after shared molecular weight (e.g. HSP10, HSP40, HSP70 and HSP90). Their function and name come from experiments on polytene chromosomes from Drosophila Melanogaster in which specific areas of high RNA transcription (chromosome puffs) were linked to the expression of heat shock proteins after subjection of the tissue sample to a temperature shock (30 °C or more) [14-16].

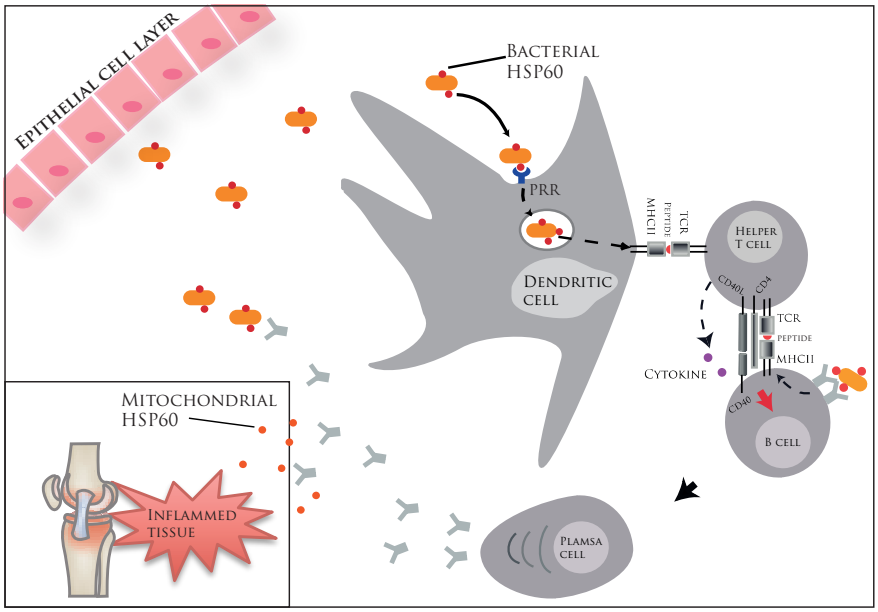


Figure 3: An overview of the immunological processing of a bacterial infection with emphasis on the development of antibodies towards bacterial and human HSP60. Pathogen-associated molecular patterns (PAMPs), such as bacterial HSP60, is recognized by a pattern recognition receptors (PRR), believed to be toll-like receptor 4 (TLR4) [5, 6, 13, 17, 18], inducing engulfment of the bacteria by phagocytosis. A peptide (8-10 aa) from the bacterium is presented by the dendritic cell in a MHC II class molecule and recognized by the T cell receptor (TCR) on a helper T cell. A Conformational epitope on a bacterium are recognized by BCR on the B cell, which leads to phagocytosis of the bacterium. The charged helper T cell delivers secondary signals in the form of CD40L and cytokines stimulating the B cell to proliferate and develop into clones of antibody producing plasma cells specific for HSP60. Such antibodies have been suggested to be cross-reactive and involved in the inflammatory response seen in joints among SpA patients.

The structural composition of the human HSP60 chaperones is best understood comparing it to “a barrel” or “isolation chamber” (see figure 1 in [13]). Generated by ATP and with affinity for hydrophobic patches, these chaperones take care of unfolded proteins needing the final touch after ribosome release. Since the human cell can be exposed to different stress situations (oxidative, metabolic & heat shock) as would be the case with inflammatory tissue the cell is engineered with an effective feedback system that responds to any increase in misfolded proteins by generating a response that upregulates the expression of HSPs. Human HSP60 has been shown to stimulate different effector components of the innate and adaptive immune system and considered to be very immunogenic with possible implications in cell damage signalling. In the literature such proteins are also named alarmins, the eqiuvelent to PAMPs (figure 3).

As a result, much research has focused on human HSPs and their immunogenic epitopes to evaluate their importance in different autoimmune diseases. Such studies include serological examination of antibody levels found towards human HSP60 using the enzyme-linked immunosorbent assay (ELISA) [13]. Development and improvement of this technique have been a main research aim in the first 2 research papers of the thesis [8, 19]. The generation of antibodies to both bacterial and human HSP60 is presented in figure 3, which as results of their homology is believed to be cross-reacitve. Such antibodies have never been demonstrated to be of pathological importance in SpA, highlighting the need for more research. Paper I investigate an association between anti-HSP60 and disease while paper II investigate an association between anti-HSP60 and disease progression.

1.1.2. IGG SUBCLASS CHARACTERIZATION

The B cells are important effector cells of the humoral immune response that, upon maturation to plasma cells, produce and release a variety of antibodies (immunoglobulins, Igs) into the blood and infected tissues. Here the Igs function as a soluble defence-mechanism that target and neutralize intruders and their virulence factors. The naive B cells, which by definition have not recognized an antigen, are equipped with B cell receptors (BCR) anchored to the cell membrane through their C-terminal. The structure of a typical Ig can be seen in figure 4A, illustrating the BCR of a naive B cell.

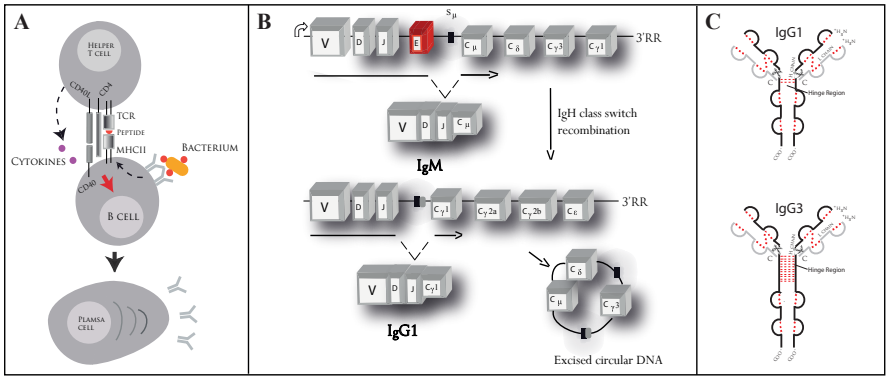


Figure 4: A) The BCR. **B)** Isotype switching of constant Hc genes. The IgM Hc can be seen as a single transcription unit of 4 exons. V: variable segment, D: diversity segment, J: joining segments, E: enhancer region, ↱: Promotor region. **C)** Biochemical structure of immunoglobulin G subclass 1 and 3 (IgG1 and IgG3).

The IgM molecule consists of 2 identical light chains (Lc) and 2 identical heavy chains (Hc), linked together by inter-chain disulfide bonds. The amino-terminal portion of the 2 chains is characterized by a highly variable amino-acid composition, which is involved in antigen binding and referred to as the complementary-determining regions (CDR). The constant region of the heavy chains holds the molecule together and is involved in several effector functions such as complement binding and binding of high-affinity phagocyte Fc receptors. The hinge region between the Fc and constant region of Fab Hc gives the Ig its flexibility. Every Ig is accompanied with a co-receptor named cluster of differentiation (CD) 79, which can be seen next to the IgM molecule in figure 4A. CD79 is a membrane bound transmembrane heterodimer, which generates a signal into the B cell after antigen recognition by the BCR. This is coordinated by immunoreceptor tyrosine-based activated motifs (ITAMs), which is located at the intracellular tails of CD79. The forces that hold together the antigen:antibody complex are of noncovalent nature and involve forces such as hydrogen bonds, hydrophobic interactions and van der waals forces. An important aspect of this binding is the fact that Igs recognize the overall surface of the specific antigen (conformational epitope) and the contribution of the different forces depends on the particular Ig and antigen. Compared to other interactions seen among proteins the antigen binding possess many aromatic amino acids, which are important for pulling together the two surfaces that are complementary in shape. B cells mature in the bone marrow where the gene rearrangement and allelic exclusion of the H and L chains leads to the cell-surface expression of the BCR. After maturation, involving many different gene-regulatory proteins, the IgM^+ B cells migrate through the bloodstream to secondary lymph nodes where they at first get localized in the follicles. If stimulated with antigen and activated by specific charged helper T cells (figure 3) the IgM^+ B cells migrate to germinal centres. Within this microenvironment IgM^+ B cells proliferate and undergo somatic hypermutation and isotype switching to create Ig diversity and increase antigen-binding specificity and avidity. This in turn leads to maturation of Ig producing plasma cells and memory B cells, which, upon a secondary infection involving the same antigen retaliates with immunity. Several of today's vaccines are constructed to encourage this maturation of memory [20].

The process of class switching involves changing of the constant region of the Ig Hcs (figure 4B). IgM is the first Ig to be secreted and expressed on the surface of naive B cells, which is a result of its position among the constant Hc gene segments. The recombination of Hc genes does not include the variable regions (CDR) and

leaves the new isotype with no change in antigenic specificity. Thus, different daughter cells from the same activated B cell are able to produce Igs of different isotypes or subclasses (e.g. IgG1, IgG2, IgG3 & IgG4). This is an advantage because the different isotypes of Igs that differ in effector functions are preferred for different antigenic stimuli. The switch factor that determines the expression of these specific heavy chain genes comes from $CXCR5^+$ charged T helper cells and involves specific receptor recognition and cytokines. Cytokines are small-secreted messenger peptides that are used for communication between cells and are essential for controlling and regulating many different cellular functions such as the germline promotors of Ig genes. CXCR5 is the receptor for CXCL13, the chemokine that attracts cells to B cell follicles. A recent study showed that B cells respond and switch to the production of Ig isotypes other than IgM more rapidly when $CXCR5^+$ $CD4^+$ T cells help them compared to naive $CD4^+$ T cells [17]. Compared to Igs the T cells (CD8 and CD4) recognize only a linear sequence of a limited amount of amino acids from the original antigen. This reveals an important aspect of the association between B-& T cells, as they do not necessarily recognize the same epitope of the pathogen. Thus, if switched Igs (IgG) are found in serum with affinity for a given epitope these Igs reflect a previous interaction with an activated helper T cell but does not imply that the helper T cell itself would recognize exactly the same epitope. It might be a sequence hidden deep within the antigen. This interpretation leads to the possibility that preserved sequences within highly conserved proteins such as HSP60 could trigger a T cell generated autoimmune response.

In humans 5 classes of Igs exist: IgM, IgA, IgD, IgE and IgG. Their genes are found in a large cluster located on chomosome 14. IgG is found to be the most abundant isotype in plasma and as a result of its small size it easily diffuse out of the blood and into tissues and extracellular fluids where it has its main function in neutralizing toxins, opsonizing pathogens and activating the complement system [21]. The IgGs can be further divided into 4 different subclasses designated IgG1, IgG2, IgG3 and IgG4. The subclasses differ in structure with special attention towards the hinge region and the position of disulfide bond linkage between Hc and Lc. In respect to the results presented in paper I and II only the biochemical properties of IgG1 and IgG3 is presented (figure 4C). The specific flexibility decreases in the order IgG3>IgG1>IgG4>IgG2 and is suggested to be a result of the higher activity of IgG3 and IgG1 in the ability to bind $Fc\gamma R$, when compared to the other subclasses [22]. Such binding trigger effector functions such as phagocytosis, endocytosis, release of inflammatory mediators and antibody-dependent cell mediated

cytotoxicity (ADCC). IgG2 does not activate the complement system and has been shown to be the dominating subclass in anti-polysaccharide responses, which is regarded as a T cell independent response [23, 24]. IgG4 is still poorly understood but largely restricted to non-microbial antigens [25]. The IgG subclass distribution in an anti-bacterial response is heterogeneous, since bacteria contain many different antigenic components with variations in protein and carbohydrate structure. Serum concentrations of the human IgG subclasses show the following pattern: IgG1>IgG2>IgG3=IgG4.

1.1.3. INTERLEUKIN-1 ALPHA - GENE, PROTEIN AND BIOSYNTHESIS

"Interleukin" as a name is given to a group of secreted proteins also refered to as cytokines. They are produced by many types of cells but first seen expressed by leukocytes (leukin) that uses them for cell-to-cell communication (inter-). Today, close to 40 interleukins have been identified, which are named with "IL" followed by one or more numbers and letters (for example, IL1A) (HGNC).

The *IL1A* gene is 11.48 Kb long and positioned on chromosome 2q14 (NCBI). It is part of the IL-1 gene cluster family neighbouring its homologue *IL1B*. Their given names reflect a history of a common ancestor (IL-1) and their trace record in the literature promotes that their products (IL-1α and IL-1β) are important inducers of inflammation [26].

Producers are primarily found among myeloid cells but keratinocytes and epithelial cells are also well-studied contributors of IL-1 production. Most importantly, however are the monocytes, macrophages and dendritic cells, which are known for their role as phagocytes and transmitters of immune responses, respectively. The precursor to macrophages is the monocyte, which is classified into the following subsets (human) based on the expression of surface markers CD14 and CD16: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$) [27]. These cells have been found to have different function in the blood circulation discussed elsewhere [28]. When the monocyte migrates from the blood to the tissue it differentiate into a macrophage, which have been divided into 2 different groups based upon the type of their activation.

Differentiation of the classically activated macrophages requires LPS, IFN-γ, or TNF-α. The alternatively activated macrophages require IL-4 and/or IL-13 as stimuli [28]. The two subsets are also termed M1 and M2, respectively [29, 30]. M1

machrophages is the source of IL-1 and the cell described in the following section (figure 5). In order for IL-1 to be produced by macrophages a stimulus of the pattern recognition receptor TLR4 is required. The downstream sequence of events is presented in figure 5A. The promotor sequence of *IL1A* contains bindings sites for a number of transcription factors including NFκB and AP-1 highlighting a very controlled but flexible regulation of gene expression. Once NFκB is translocated to the nucleus it binds the promotor via the general consensus sequence (ggg RNN YYC C, R=purine, Y=pyridine) transcribing the pro-inflammatory cytokines TNFα, pro-IL-1 and IL-6.

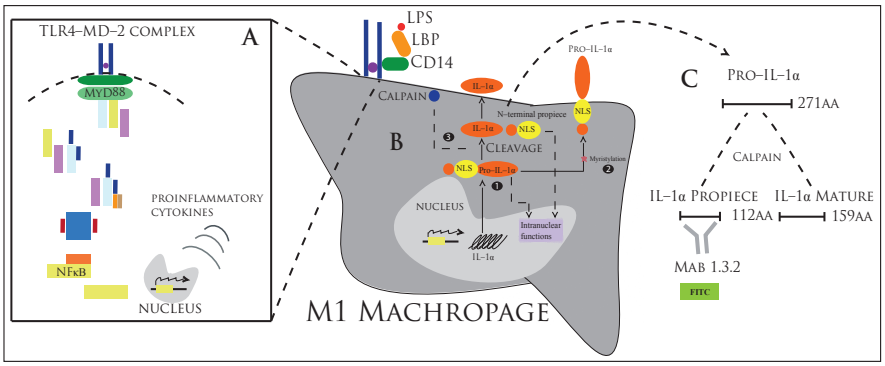


Figure 5: Signal transduction and processing of IL-1α in a M1 macrophage. **A)** The binding of LPS to TLR4 on the surface of the macrophage forms a heterologous receptor complex. Anchored to the membrane CD14 and LPS binding protein delivers LPS to the TLR4-MD-2 receptor creating the TLR4 dimer. The TLR4 dimer attracts the adaptor proteins TIRAP and MyD88. These adaptor proteins interact with TLR4 by TIR domains while MyD88 interact with partners through death domains. A box is highlighted to the right in which the signalling cascade is outlined. The final part of the transduction presents dissociation of IκB (orange) from NFκB allowing it to enter the nucleus where the transcription of proinflammatory cytokines takes place including pro-IL-1α mRNA. **B)** Pro-IL-1α processing. **1)** Pro-IL-1α is able to translocate to the nucleus without any modification. **2)** Pro-IL-1α can be myristylated and translocate to the plasma membrane. **3)** Pro-IL-1α can be cleaved by the membrane-associated enzyme calpain to an N-terminal propiece and mature IL-1α. Mature IL-1α is released and the N-terminal propiece translocates to the nucleus. **C)** In our study we generated a monoclonal antibody towards the precursor of IL-1α and conjugated the antibody to FITC presented in paper IV [26].

The translation of IL-1α makes a protein of 271 aa (Figur 5C), which along with IL-1β contains no ER- or Golgi targeting sequence and hence secreted by an unconventional protein secretion pathway. Both proteins are processed into biological active parts by cleavage. IL-1α is cleaved in mice by calpain, a calcium-dependent cysteine protease resulting in a nuclear-associated 112 aa propiece and a 159 aa mature and secreted version (figure 5B). In comparison with IL-1β, the

cellular mechanism of IL-1α secretion still miss consensus among researchers. Recent discoveries by two independent groups Fettelschoss *et al.* (2011) and Groß *et al.* (2012), however, have made important progress [31, 32]. Both groups agree that the secretion of IL-1β depends on proteolytic cleavage by caspase-1, which is activated by a pathogen-sensing multiprotein complex referred to as the inflammasome (a very detailed visualisation of these steps can be found in the review of Dinarello *et al.* (2009), figure 1) [33]. However, more difficult is it to agree on the dependency of the inflammasome in IL-1α secretion, which has resulted in a follow-up comment on the two independent research results by Yazdi *et al.* (2012) [34]. In the paper of Fettelschoss *et al.* (2011) they find evidence to support that IL-1β acts as a chaperone for IL-1α secretion. Furthermore, they discuss that TLR activation is sufficient for the production of surface IL-1α, whereas the secreted version needs additional activation of the inflammasome, most notably NLR family, Pyrin domain-containing 3 (NLRP3). In order to activate the complex the researchers made use of uric acid crystal and nigericin. Working with the same research question, the group of Groß *et al.* presented an inflammasome-independent secretion of IL-1α via crystalline activators. These findings have demonstrated a need for fourther research to identify the specific circumstances by which IL-1α is secreted and cleaved.

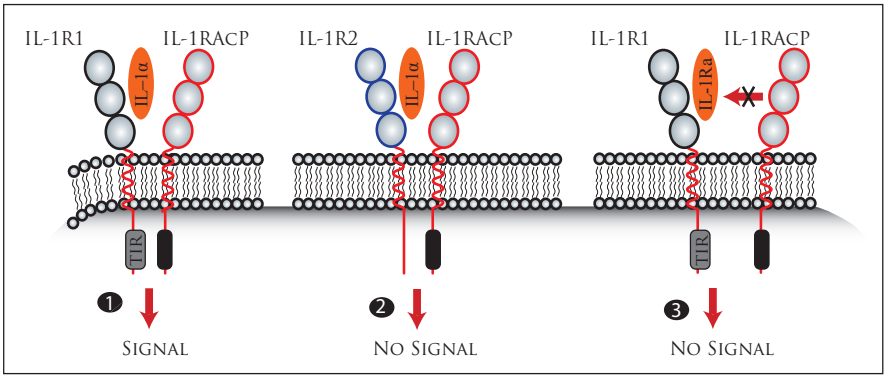


Figure 6: Signal- or no signal transduction for IL-1. **1:** IL-1α binds to IL-1R1 recruiting IL-1RAcP. The binding forms a heterodimeric receptor and triggers the event outlined in figure 5A. **2:** IL-1R2 is a decoy receptor, which can also bind IL-1α but does not recruit the interleukin-1 receptor accessory protein (IL-1RAcP). Hence, no signal is transmitted to the inside of the cell. **3:** IL-1Ra is an IL-1 receptor antagonist that upon binding to IL-1R1 prevents the recruitment of IL-1RAcP [33].

The reason why it could come as a surprise that the related cytokines, IL-1α and IL-1β, have different biological functions is demonstrated in their affinity for the same

receptor IL-1R (Figure 6,1). Furthermore, the proteins are related by gene organization, sequence similarity and gene organization. In order to control the system several mechanisms have been found in which IL-1 signaling is blocked as outlined in Figure 6,2. This makes good sense as the multiple roles for IL-1 present itself in disease-related biological processes including inflammation, wound healing, metabolism, immunomodulation and the endocrine system [35]. Therefore, a defect in the system would quickly manifest itself in clinical symptoms, such as fever and rash.

1.1.4. INTERLEUKIN-1 AND DISEASE

The understanding of the genetic defect in IL-1-associated diseases improved with the finding of a gain of function mutation (mutation that confers new or enhanced activity on a protein) in the gene that encodes the inflammasome (*CIAS1*) [34, 36, 37]. Diseases include familial cold autoinflammatory syndrome, Muckle-Wells syndrome, gout, type II diabetes, inflammatory- and autoimmune diseases. Many of them have been treated with the IL-1 receptor antagonist anakinra (figure 6,3) with a positive clinical outcome but most notably rheumatoid arthritis (Ra). Ra is considered an autoimmune disease with uncontrolled inflammation in synovial membranes of the small joints of the hands and feet. Furthermore, they have an accumulation of mononuclear phagocytic cells in the lining layer, which presents the close contact between producer of IL-1 and inflammed tissue [38]. Arthritis as a medical term, however, is differentiated into a large number of diseases with differential diagnostic issues in the clinic and hence brought the interest of IL-1 genetics into many athrogenic diseases. Sequentially, the search for a genetic defect has been aligned with next generation sequencing technology producing a number of genome-wide association studies (GWS) that links SNP's to the development of disease. Such studies have recently (<10 years) been published in which the diseases of SpA have been linked to SNP's in IL-1α.

These findings present investigations in which a statistical approach is used to compare sequencing results of genetic material from diseased and healthy volunteers. Maksymowych *et al.* (2006) finds rs3783526 (*IL1A*) to be associated with SpA [39]. Sims *et al.* (2007) finds strong association with three single nucleotide polymorphisms (SNPs) in the *IL1A* gene (rs2856836, rs17561, rs1894399) [40]. In the Monnet *et al.* study (2012) the SNP rs2856836 and rs1894399 in *IL1A* was significantly associated with spondyloarthritis and AS,

respectively [41]. Finally, The Lea W-I *et al.* (2012) study finds that the OR of the 2 allele of *IL-1A* + 889 (rs1800587) was found to be significantly increased in Europeans with AS [42].

Together with IL-33 the IL-1α protein is described in the litterature as a dual function cytokine explained by the biological activity of both cleavage products. The role of nuclear association is believed to be part of a mechanism to alarm the immune system upon cellular damage. The same has been applied for human HSP60 as autoantibodies are found in healthy individuals and increased in a number of diseases with an inflammatory pathogenesis. Hence, as for IL-1β, many years of research have studied the biological processeing of inflammatory cytokines as they present potential targets for the development of immune-modulators such as the IL-1 receptor antagonist (IL-1Ra) analog Anakinra (figure 6,3).

Results from studies investigating the effect of Anakinra as therapeutic for treatment of SpA have not been encouraging and with different outcome. In a study by Tan *et al*. a 61% reduction in spinal and/or sacroiliac joint MRI inflammation was observed [43]. Hence, the conclusions so far, based on open studies, are that Anakinra is mildly efficacious. However, as 20-25% of patients do not respond to Anti-TNFα treatment alternative options are needed. Such alternatives could be blocking for inflammatory cytokines such as IL-6 (tocilizumab) or IL-17 (Secukinumab). In comparison to IL-1β, IL-1α have been found to function both as a secreted and as a membrane-bound cytokine and hence, can trigger and sustain inflammation locally, especially in tissue of dying and necrotic cells. This is maintained by the induction of several substances such as phospholipase A2, cyclooxygenase-2, and nitric oxide causing pyrexia, hypotension, and induction of acute-phase reactants. The different mechanism of action between the two cytokines may hide alternative treatment strategies. Therefore, further research is needed to elucidate the mechanisms of production for IL-1α among inflammatory cells and if such measurements could elucidate a role for IL-1α in SpA. A first mover would be to create an assay usable for an investigation of a possible functional link betwenn IL-1α SNP's and induction of IL-1α in humans. The association of IL-1α propiece with the nucleus have created a window for a new indirect aproach to determine the kinetics of IL-1α in human monocytes outlined in paper IV[26].

2. METHODOLOGICAL APPROACHES

The 3 main methods used in this thesis will be described in the following sections.

2.1 INDIRECT ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) is the preferred solid-phase assay for clinical serology studies with a large sample size. The assay is carried out with a few steps (figure 7) and positivity can be visualized from the development of colour in a 96-microwell plate, which is measured with spectophotometry.

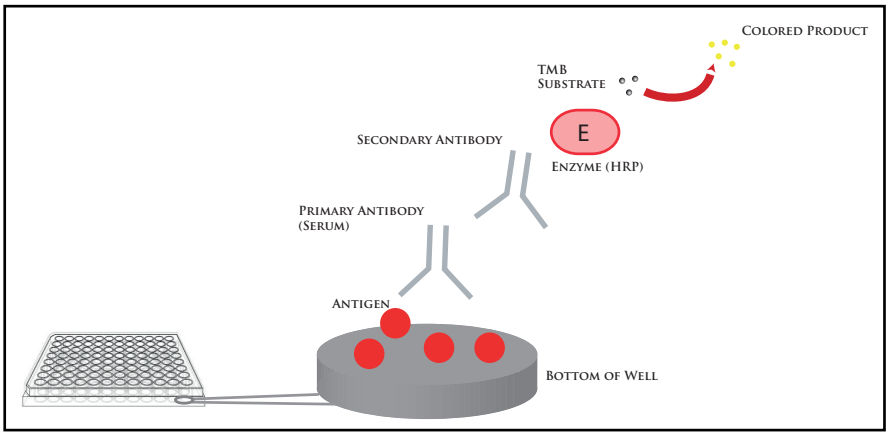


Figure 7: Antigen is targeted with antibodies from the serum before being detected through an enzyme-linked antibody that turns a substrate into a readable coulor, which is converted to optical density units.

To determine the presence of specific antibodies, such as the subclasses of IgG, the preferred strategy is referred to as indirect ELISA (figure 7). The assay can measure antibodies produced in vivo in humans and therefore reflects the outcome of a natural, human immune response against a given antigen. This strategy was adopted to measure antibodies in serum from the SpA patients. The time-consuming part is the need of optimization between different patient groups. Therefore it is important to understand how the different components of the assay reflect the final optical density (OD) reading [44]. One of several considerations would be the immobilization of antigen, which bind to the UV treated surface of polystyrene. A high concentration of antigen may not allow antibody to bind through steric inhibition. The easiest way to access this is by making a so-called chessboard

titration (CBT) where serum that contains antibodies against the antigen is diluted to make titration-curves for different antigen concentrations [44]. This enables the practician to choose the best concentration because a plateau from the OD readings would indicate when antigen is in excess. The best concentration of antigen is where the OD level decreases as soon as the serum is diluted creating the most sensitive assay. The same applies when the concentration of enzyme-conjugated antibody is evaluated (figure 8).

Because the primary antibody (serum sample) would be measured incorrectly if antigen or enzyme-conjugated antibody were in deficit we choose to add a high dilution of antigen (4 µg/ml), and then decided the dilution for the enzyme-conjugated antibody. In figure 8 an example of titration curves is shown (unpublished). The black and blue dotted gray lines have reached a plateau at low serum dilutions (1/20, 1/40 and 1/80) indicating that a lower concentration of enzyme-conjugated antibody is needed. A dilution of 1/20000 has an optimal titration as it declines as soon as the serum is diluted and thereby giving the assay high sensitivity.

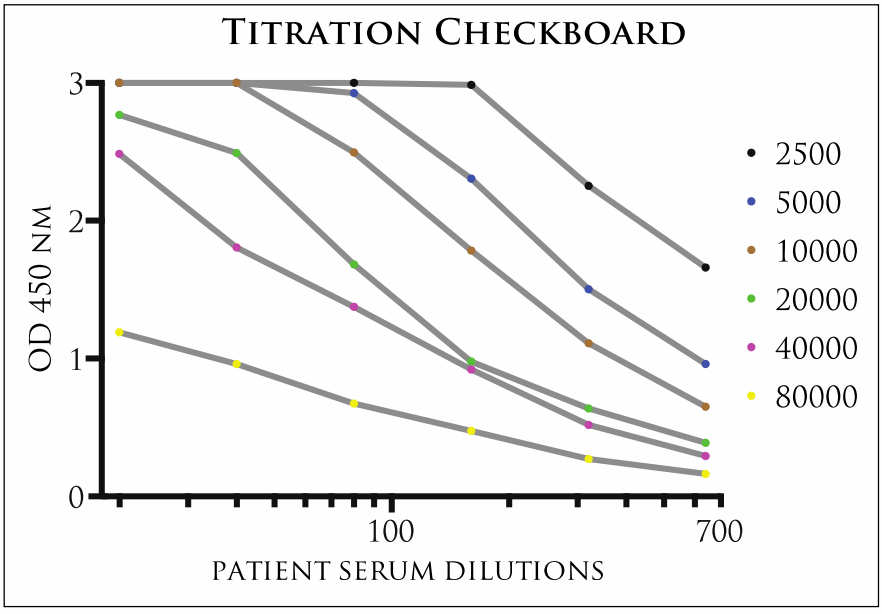


Figure 8: Titration curves used to choose the optimal concentration of enzyme-conjugated antibody (see text). A serial double dilution (1/20 – 1/640) of serum from patient 81 (Study I), who is seropositive for *C.jejuni* HSP60, was treated with different concentrations of enzyme-conjugated sheep-anti-human IgG1

(1/2500 – 1/80000). The plate was coated with 4 μg/mL *Campylobacter jejuni* HSP60. The X-axis is log10 transformed.

The CBT measurements can be evaluated using serum from a patient from the cohort with the highest OD reading. This ensures that the chosen concentration of enzyme-conjugated antibodies maintains the OD-readings for the entire patient material below the limit of detection set by the spectrophotometer. Because the measurements have to be quantified, the chosen curve should give an OD level that corresponds to a dynamic area of the standard curve (i.e. before it reaches a plateau). Usually an OD around 1,5-1,7 is preferred.

To quantify the measurements of IgG content (ng/mL) in serum the OD of the sample can be interpolated into a standard curve (an example of this is present in paper II [19]), which is typically a serial dilution of the target. In this case Nunc™ Maxisorp plates were coated with dilution series of native IgG1, IgG2, IgG3 and IgG4 from human myeloma plasma in CCB buffer (100 mM $NaCO_3$, pH 9,6).

Every plate is made with two blanks (only buffer), representing unspecific binding. The mean value of the blanks plus two times the standard deviation (SD) was subtracted from every patient sample measurement, which represents limit of detection. Patient and control samples were placed alternatively together in the setup, so in every plate both patients and controls were analysed. This prevents a bias between the patient-group and the control group caused by plate variation. Bias due to inter-assay variation, which is normally 5-15%, was reduced by adding a control sample on each plate, hereby quantifying the variation between different plates from different days.

2.2 ISOLATION OF MONOCYTES/MACROPHAGES

The ability to isolate the cell of interest is often the first step to overcome when human blood is being studied and has been a major part of the scientific process in the last paper of this thesis. The isolation of mononuclear cells based on the principle of centrifugation dates back to 1968 [45]. The discovery was made by Dr. Arne Bøyum and the commerzialisation of the principle have been named lymphoprep™. The medium has a density of 1.077 g/mL, which upon centrifugation seperates monocytes and lymphocytes (peripheral blood mononuclear cells (PBMCs), < 1.077 g/mL) from other components of the blood such as polymorphonuclear cells and thrombocytes (figure 9B). In order to get the best

separation of cells the preperation of the two layers (i.e. blood and lymphoprep™) must be done with caution so the layers do not mix before centrifugation (figure 9A). Hereafter cells can be removed from the interface, washed and transfered to medium for culturing.

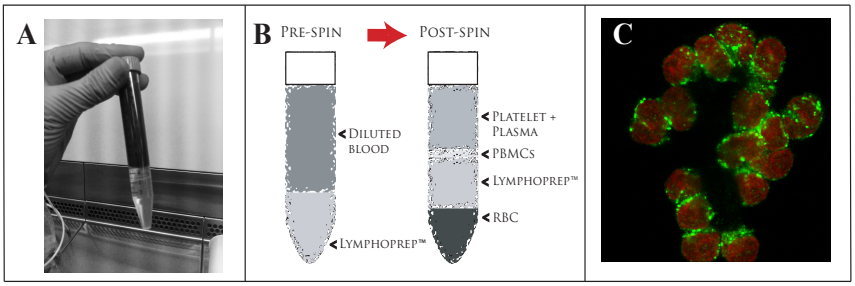


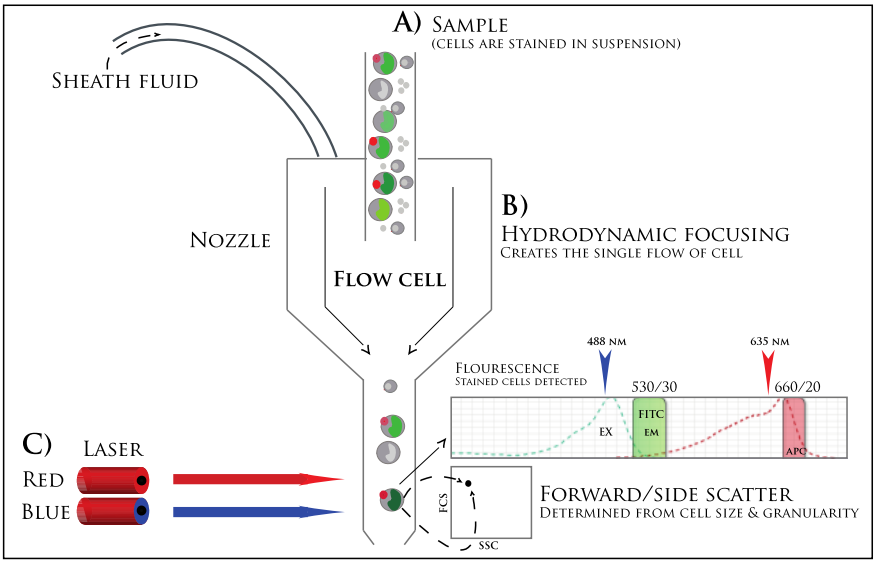
Figure 9: A) Preperation of blood with lymphoprep™ before centrifugation **B)** Illustration of the impact of centrifugation on the different components of blood **C)** Microscopy image of machropages with a red nuclear stain and a green stain for CD68 (modified from [26]). Microscopy was performed on a SP5 confocal microscope (Leica) with a CX PL APO 100x/1.47 OIL CORR TIRF objective.

Monocytes adhere to plastic, which is fueled by the prescence of serum in the medium. The prescence of extracellular proteins (such as fibronectin) in serum creates a surface that attracts specific surface proteins of the monocytes promoting attachment and simulating the in vivo migration of monocytes into the tissue initiating machrophage differentiation [46]. This cellular trait can be used for immunostaining (figure 9C) to identify markers valuable for cell identification or viability. Such a method was used for the identification of intracellular localization of IL-1α propiece in human macrophages in paper IV [26].

2.3 FLOW CYTOMETRY

The explanation of this method is in the name as cells (cyto) in motion (flow) is being measured (metry). The technology is best understood with a view of the flow cell, in which the sample is injected at the start of a run (figure 10). An important part of a succesful read of a complex cell sample is the ability of the flow cell to make cells pass the laser/s one by one. This is accomplished from hydrodynamic focusing, a technique that alters the velocity of the central fluid of the sample by passing faster moving sheath fluid on each side of the central core (figure 10B). Leaving the flow cell stained cells are then illuminated with a laser that scatter upon impact. The scattering is dected at two angles that measure cell size (<10 degrees) and granularity (90 degree) also refered to as forward (FSC) and side scatter (SSC)

(figure 10C). Furthermore, fluorochromes on cells are detected as they get excited from the specific wavelenght of the laser and emit light at another wavelength. Emitted SSC and fluorescence signals are diverted by mirrors and optical filters to the photomultiplier tubes (PMTs) and a photodiode collects the FSC signal. By this, optical signals are converted into electronical signals (voltage) and finally into a digital number by the analog-to-digital converter (ADC) that can be visualized and analyzed with computer software. With the ability to detect up to 20.000 cells/sec and the inclusion of multiple lasers in newer flow cytometers and an even more so incomprehensible amount of different fluorochromes has made flow cytometry a favourite method of choice for cellular research [47].



Figur 10: The flow cell. A) A sample, such as lymphoprep™ isolated PBMC's, are cultured, stained and kept in suspension. **B)** Hydrodynamic focusing is a technique that creates the single flow of cells important for accurate measurements (see text). **C)** Cells scatter light when they pass through lasers beams. The level of light scattering in straight (forward) direction is proportionate to cell size. Cells also include granules, compartmented nuclei and other organelles. This leads to side scattering of light. Side scattering depends on cell structure (e.g. granularity). A diagram of excitation and emission spectrums of common fluorochromes is presented created from BD fluorescence spectrum viewer. This is a nice tool to validate the chooise of fluorochromes before staining. Sometimes when polychromatic flow cytometry is needed potentiel overlap of spectrums between fluorochromes requires compensation to remove false positives [48].

A typically cytogram of common blood cells is presented in figure 11A. The higher up the y-axis the more side scatter and hence granularity of the cell. The same

applies to cell size forward scatter located out the x-axis. As lymphoprep™ is used for isolation only lymphocytes and monocytes are expected to be present from a sample. This is visualized from the cytogram in figure 11B where polymorphonuclear cells with high granularity are excluded. If enough cells are collected it is possible to locate the different populations, which in data software, such as FlowJo™, can be gated out of the total cell sample for further analysis of specific cell markers (figure 11B). For monocytes this could include a panel of CD14, CD16, CD68 or CD163 [30]. Even though the flow cell is equipped with the laws of physics in terms of hydrodynamic focusing cells that stick together could stil be an issue of concern. Therefore it is recommended to make a FSC-W vs. FSC-A plot to remove aggregates to improve the quality of analysis. The principle is explained and visualized in figure 11C.

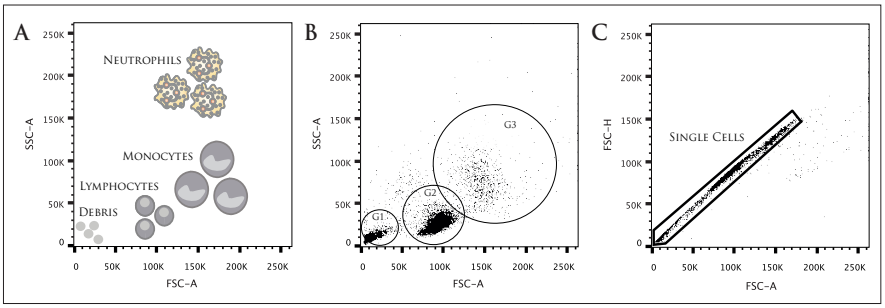


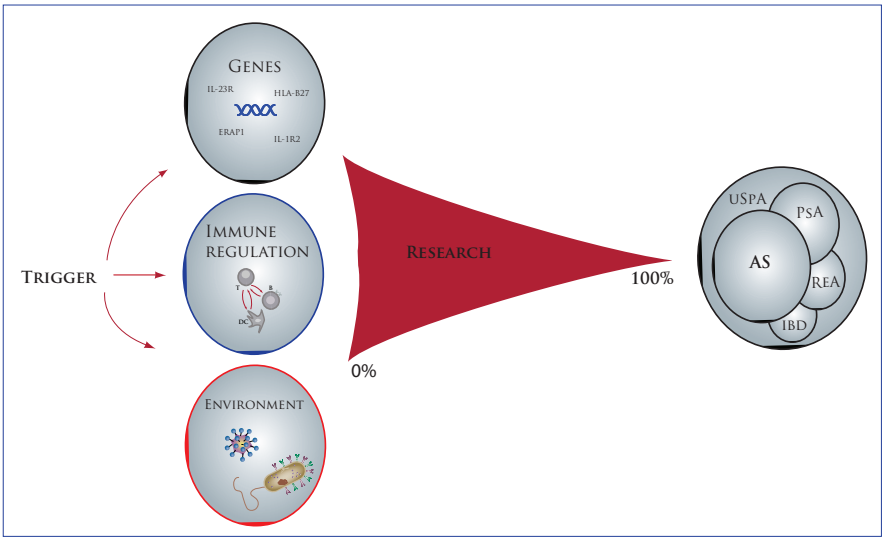
Figure 11: A) The placement of cells from blood in a forward (FSC-A) and side scatter (SSC-A) diagram also refered to as a cytogram **B)** Gating (G1, G2 and G3) for population of cells using FlowJo™. **C)** Doublets is removed by a FSC-W vs. FSC-A plot before data analysis (unpublished data). A pulse is generated when cells enter the laser beam and the width of this pulse presents the time the cell is in the beam and therefore an indicator of cell size. Resolving singlet cells from aggregates is based on the fact that aggregates are larger than single cells and will have a greater pulse width signal than singlet cells. These aggregates can be seen to the right of singlet cells in a FSC-W vs. FSC-A plot.

The remarkably speed of flow cytometry is overt. However, the information from a flow cytrometric analysis comes with limitations. This can be explained from the principle of fixation, part of the staining protocol. The most commonly fixative for cells is formaldehyde. The effectcs of formaldehyde is produced from its ability to react with side chains of proteins to form reactive hydroxyl-methyl groups. These reactive complexes may combine with each other forming methylene bridges or cross-links, preserving or fixing the antigenic structures in the cell important for staining purposes. However, as cells are fixed the window for analysis only concerns the cell state at the fixation point. Therefore, a peek into the behaviour of a specific subset of cells (e.g. activated T cells) in time and space is limited. Today, the ability

to track the polarization of the immune response is based on the identified cells and cytokines produced from the isolated biological material under investigation. However, when a sample derives from tissue with chronic inflammation some of the identified cells may be present with a phenotype that fluctuates at levels unsuitable for fixing chacterisation. In other words, the in vivo polarization of a single cell in that network would be destroyed and so would the analytical significanse. Approaches to circumvent this issue is the development of single cell technologies reviewed elsewhere [49].

3. SUMMARIZING DISCUSSION

The intention of this thesis was to look for a triggering agent that would explain both the bacterial infections and specific genes involved in the predisposition to SpA. Therefore, bacterial HSP60 and the genes *HLA-B27* and *IL-1A* was investigated with emphasis on immunological dysfunction. Antibodies to bacterial HSP60 and human HSP60 were compared and an indirect method for IL-1α quantification in monocytes was conducted [8, 19, 26]. Hence, this work include information on both adaptive (cross-reactive antibodies) and innate immune responses (macrophages) belived to be of importance in SpA pathogenesis. Based on this thesis the hypothesis of molecular mimicry could not be supported and groundwork for a functional analysis of SNP's in *IL-1A* was demonstrated. However, the missing link that explains the association between genes, immune regulation and the environment responsible for SpA pathogenesis is a massive challenge (figure 12).



Figur 12: A visualization of the complex research path of SpA pathogenesis.

The products of the two genes highligted in this thesis respresent different molecular functions, however the potentiel consequences of their dysfunction is obvious. HLA-B27 molecules present peptides to CD8+ T cell and if peptides are presented abnormally it may trigger an inflammatory response. IL-1α is a proinflammatory cytokine that with a defect in it's thightly controlled expression system, could cause

damage to sorrounding tissue (1.1.3). How these components including Anti-HSP60, HLA-B27 and il-1α could make up a combined pathology for SpA is difficult to imagine. It would suggest that a defect HLA-B27 molecule (MHCI) breaks it's own role for cell specificity and presents a peptide of HSP60 to an autoimmune prone CD4+ T cell that upon recognition travels to the germinal center and activates a B cell with affinity for HSP60 to make antibodies. Aligned with this the CD4+ T cell would also produce INF-γ that activates tissue macrophages to produce IL-1α. Supporting the ability for HLA-B27 to present larger peptides mimicking a MHCII molecule comes from a study by Urban *et al.,* (1999). With the use of a monoclonal antibody (MARB4) towards a subset of HLA-B27 molecules they investigated the length of bound peptides by mass spectrometry and Edman sequencing. Here they actually observed the binding of larger peptides (>9 aa) by HLA-B27 subsets. However, further analysis in a transfected cell line with a defective TAP1/TAP2 complex (protein transportation) suggests that MARB4 recognize peptides destined for further trimming as HLA-B27 detection was abolished [50].

The biological properties of the HLA-B27 molecule has been investigated exstencively with special attention to antigen presentation and the so-called ”arthriogenic” peptide among the B27 allele family [51]. However, the development of a spontanous inflammatory phenotype in b27/human β2-microglobulin mice knocked down for CD8+ T cells have generated noise on their importance in SpA pathogenesis [52]. This said, newer studies have shown both genetic assocciation of SNPs in CD8+ T cell genes (*RUNX3*) and their precense in high number in synovial fluid of PsA patients suggest that mouse as an inflammatory model may be inapplicable [53-55]. Three decades have passed and the mystery of *HLA-B27* still exists. However, improvements in technologies that create and combine both genome and proteome measurements on biological systems including next generation sequencing (NGS), polychromatic flow cytometry and mass spectrometry creates more analytical power, which looks very promising for the future [49].

3.1 ALARMINS

The term alarmins was introduced at a meeting on danger signals in Milano 2006 by Joost Oppenheim [56]. Both HSPs and IL-1α are found on the unfinished list of alarmins and their primary roles in mammalian physiology as chaperones and pyrogen have suggested that evolutionary forces can reuse old proteins for new purposes. Hence, exploring the immune system from an evolutionary perspective

can be very useful for it's understanding. Many processes such as the maturation of lymphocytes and the modification of antigenic specificity among receptors of the adaptive immmune reponse arise from the principles of evolution stating that a selection for best fitness improve the gene pool. The need for this process is obvious as the change of genes among bacteria and viruses apply to the same principle. This evolutionary arms race between the mammalian cell and microbes have had and still have a huge impact on the cellular processes that are being explored by invading microbes. In this context one would argue that the development of subclasses for IgG have evolved to combat increased diversity of bacterial antigens. More so, to indirectly protect the mechanisms of tolerance and reducing the negative effects of cross-reaction. Applying the same theoretical approach to IL-1α is discussed in the next section.

3.2 CELLULAR SECRETION PATHWAYS

Looking at diversity in cellular activites from an evolutionary perspective also applies to the lack of knowledge on secretion of proteins not destined for the classical pathway. Such proteins including IL-1α lack a hydrophobic N-terminal sequence that allows entry to the ER-golgi pathway. The quistion arises "why do mammalian cells actually need additional secretory mechanisms besides the classical pathway?" [57]. Some of these proteins include angiogenic growth factors, inflammatory cytokines (such as IL-1α) and components of the extracellular matrix, which regulate cell differentiation, proliferation and apoptosis. All these functions could be considered to have important roles for cellular responses to environmental change. As the primary role for IL-1α in monocytes is to activate an immunological response upon recognition of PAMPs, it would seem very likely that such a response would be selected for by its speed and their biogenesis would therefore not be suited for ER and golgi trimming (figure 15). That said, not much research exists on the kinetics of protein secretion from nonclassical pathways and to what extend they exceed the secretion kinetics of the classical pathway. A study by Hirschberg *et al.* 1999 made measurements on protein movement in a live COS cell (fibroblast cell line from monkey kidney tissue) by fluorescence recovery after photobleaching (FRAP) [58]. The method makes use of a fusion protein tagged with GFP. The investigated protein was a mutant of the stomatitis virus glycoprotein, which can be accumulated in the ER at 40 ºC and released to the secretory pathway be shifting the temperature to 32 ºC. During the FRAP approach, a small area of the fluorescent sample in the region of interest is permanently photobleached by intense laser

illumination and the cells monitored over a period of time. If the protein in question is immobile then the bleached area will remain dark as there is no protein movement to replenish the signal. If however the protein (or a fraction thereof) is mobile, then unbleached mobile fluorophores from outside the bleached region will move/diffuse into the area and bleached proteins may in turn move out of the dark area. The rate of this recovery is a function of the protein, enabling the kinetics of movement to be calculated.

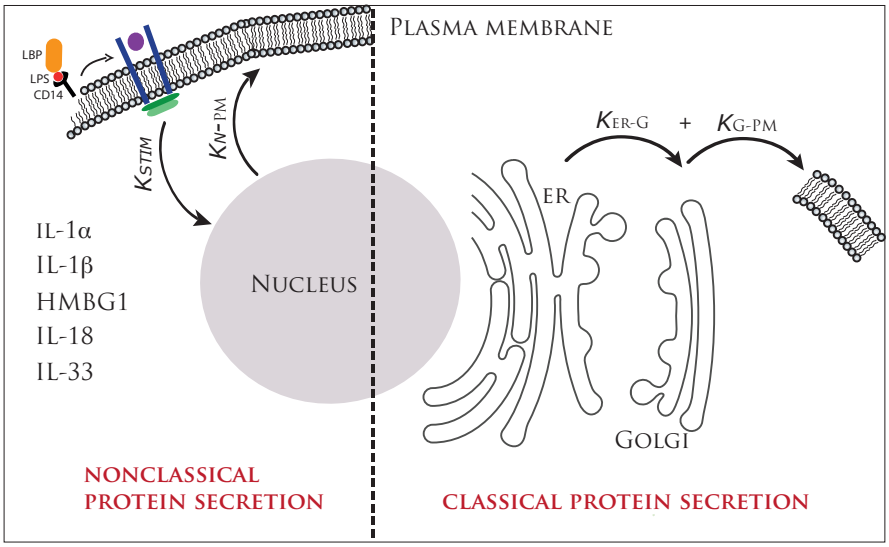


Figure 14: The difference between processing steps involved in the classical and nonclassical pathways indicate the need for more modification of proteins destined for the ER and golgi (G). Some known proteins not destined for the classical secretion pathway has been presented to the left. *K*: represents a rate by which either stimuli (STIM) or molecules is transferred from one compartment to another. PM: plasma membrane.

From this study mean rate constants for ER-golgi transport was 2.8%/min whereas golgi-plasma membrane transport was 3%/min wheras recidense times in both the ER and golgi was estimated to 40 minutes. The percentages represents the fraction of molecules transfered pr. unit time, specifying the fraction of the content in one compartment (i.e. ER) that flow to a downstream compartment (i.e. golgi). These results, allthough not from a human cell, indicate that time needed in the ER and golgi apparatus exceed what would be possible to proceed prior to the response measured for IL-1α. Furthermore, these protected secretion mechanisms opens a window for important targets that could be of interest to biomedical research. It also highligts the fact that other parts than transcription and translation may be affected

by mutations in *IL-1A*. Such parts could include secretion pathway/s of IL-1α in which the engagement between mature IL-1α and transporter proteins is affected.

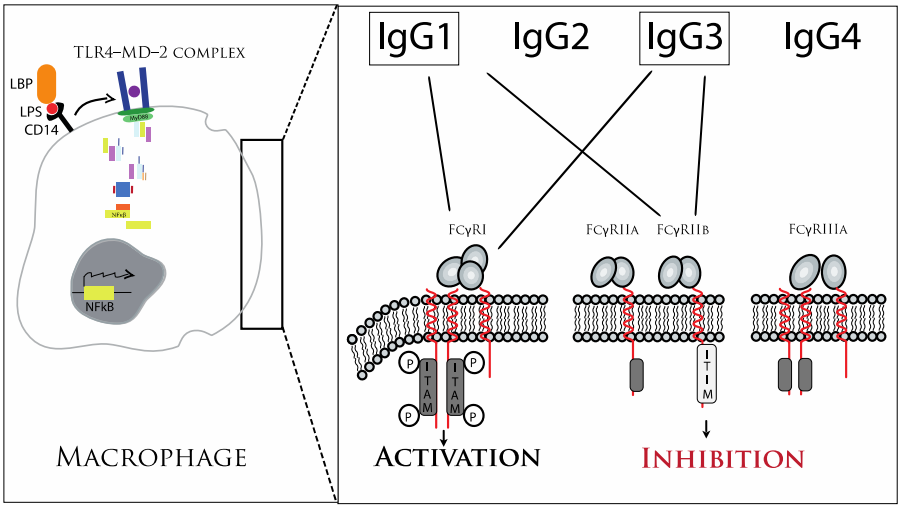
3.3 SINGLE NUCLEOTIDE POLYMORPHISM IN SPA

In line with the affordability of next-generation sequencing (NGS) methods studies that explore associations between gene variants and specific disease traits have exploded. These so-called genome-wide association studies have generated a lot of data on the genetic complexity in SpA disease. In one such study endoplasmic reticulum aminopeptidase 1 (ERAP1) have been shown to be associtated with AS in *HLA-B27* positive patients only [54]. As ERAP1 is involved in the trimming of peptides for the groove of the HLA-B27 molecule it suggests a gene-gene interaction in which the stability of peptide presentation could be affected by SNP's in *ERAP1*. However, as for *IL-1A*, it remains to be proven if such small risk fractions (compared to that of *HLA-B27*) can be associated with a functional phenotype in disease. However, from an imunological point of view SNP's in *IL-1A*, could have an effect on the regulation of both release and shot-down of secreted IL-1α with dramatic consequences for the sorrounding tissue.

3.4 IMMUNE REGULATION

Chronic inflammation is the pathological description that drives the symptoms observed among SpA patients. Even the triggering diseases such as psoriasis and inflammatory bowel disease (IBD) that lead to SpA has inflammation as the first symptomatic clinical criteria. Therefore, whatever may be the trigger, it intervienes with the tolerance or balance of the inflammatory response. Hence, understanding the regulation of inflammation is crucial. Both human HSP60 and IL-1α propiece are found within the cell at normal homeostasis. Human HSP60 within the mitochondria and IL-1α propiece in the nucleus. As they appear in the extracellular environment both of them give rise to autoantibodies, which have been observed to be elevated in a number of inflammatory diseases [13, 59]. However, none of these findings have given any diagnostic advantages to SpA patients and a role as unharmful alarmins of cell damage seems most likely [56]. The generation of anti-HSP60 and anti-IL-1α T and B cell epitopes in healty subjects prior to the generation of autoantibodies quistions how the immune system maintain the peripheral tolerance (figure 3). It is argued here to be a combined inhibitory action of T_{reg} cells and the FcγRIIB, which is expressed on both monocytes and macrophages. T_{reg} cells regulate immune responses and are regarded as the primary

mediators of peripheral tolerance. Mechanisms of action include inhibitory cytokines, suppresion by cytolysis, suppresion by metabolic disruption and finally suppresion by modulation of dendritic-cell maturation or function reviewed elsewhere [60]. FcγRIIB, not to be confused with CD16 (FcγRIIIA and FcγRIIIB), has inhibitory function acting through its immunoreceptor tyrosine-based inhibitory motif (ITIM) (figure 14). These mechanisms hide targets for theraputic interventions and hence the ability to control inflammation and autoimmunity. The IgG1 and IgG3 subclasses that have been shown to target human HSP60 in this thesis is the preffered antibodies recognized by FcγRIIB [61-62]. Questions arise, however, if these mechanisms have tresholds and what these are. Such threshold values could represent a saturation point for the regulatory component and clear the path for autoimmunity.



Figur 14: A view of the different Fc receptors expressed on monocytes and macrophages (modified from [61]**).** There are four activating FcγRs expressed on monocytes/macrophages: the high-affinity receptors FcγRI, which can bind monomeric IgG, and two low-affinity receptors (FcγRIIA, FcγRIIIA and FcγRIIIB), which are restricted to bind immune-complexed IgG. FcγRIIB is the only inhibitory FcγR. It is a low affinity receptor that binds immune-complexed IgG that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain. The cross-linking of FcγRIIB by immune complexes results in ITIM phosphorylation and inhibition of the activating signal [61-62].

3.5 CONCLUDING REMARKS

The ability to diagnose SpA as early as possible is very important for the prognosis. The earlier a diagnosis is given to the patient the earlier treatment can begin and

hence reduce the amount of damage inflammation can cause to joints and ligaments. This is the primary goal and challenge that rheumatologists face with SpA. Historically, this challenge has produced many modifications of the criterias used for diagnosing SpA dating back to the Rome criteria in 1961. The hallmark for the diagnosis of axial involvement in SpA is radiography of the sacroiliac joint. However, this method has been shown to be insensitive to early bone and joint damage. With the introduction of magnetic resonance imaging (MRI) the ability to visualize early structural changes have increased diagnostic sensitivity and improved the usuabilty of anti-TNF treatment. Such measurements were included in paper II in order to track disease activity of the patients thereby also improving the robustness of the clinical characteristics [8]. However, these procedures (MRI scans and anti-TNF treatment) require resources and come with great costs to society. In this context a disease marker traceable from urin, faeces or blood able to differentiate the subgroups of SpA would be diserable and give a better and targeted treatment.

In this thesis, a disease marker has been searched for among subclass antibodies in serum to bacterial and human HSP60. The marker would exist because of molecular mimicry stating that similarity in the proteins would give rise to an inapropiate immune response to human HSP60 and hence explain the chronic inflammation in the joints of SpA patients (1.1.1). Unfortunatly, these levels of antibodies, in combination with clinical parameters, did not have the capacity to differentiate between SpA patients and healthy donors. However, knowledge from the studies in this thesis still contributes to the analysis of cross-reactive antibodies in SpA pathology. Because of the subclass differentiation that exist between bacterial and human HSP60 the hypothesis of molecular mimicry cannot be supported.

A marker does not need to be expressed; it could just as well be hidden in the genetic setup of patients, such as *HLA-B27*, which is found among 90% of AS patients. With the development of NGS more than 10 genes with SNP's have proven to be significantly associated with SpA including *IL-1A* from GWS. Some of these genes concern inflammation (*LTBR-TNFRS1A*, *IL23R* and *IL-1A*) and apoptosis (*CARD9*) while others concern peptide trimming (*ERAP1*) and protein transport (*KIF21B*). Solving the mystery of SpA pathogenesis in the future will most likely come from research that quistions how this "trace puzzle" fit together.

4. AIMS OF THESIS

At the opening of the Danish parliament 7.9.2014 the former Danish Prime Minister Helle Thorning-Schmidt gave a speech from which the following section has been extracted and translated into English:

I received an email from a man of 49 years who suffer from arthritis of the spine. It is a terrible and debilitating disease. Medications can relieve some of the pain. It costs 30,000 dollars a year, and as he writes, he will not have to pay, "because I'm so lucky to be a Danish citizen." He also writes that it took too long before he got his diagnosis. So long, that the disease spread so much that even the best medicine cannot fix what has already been destroyed. And therefore the 49-year-old man wrote in his email to me: "It is extremely important to diagnose much earlier than in my case."

The diagnosis of this particular disease is very likely to be AS and the patient highlights the challenges that rheumatologists are dealing with in the clinic. Furthermore, it also implies why it is important that more research is conducted on the pathogenesis of SpA. That being said the overall objective of this thesis is to improve the understanding of the underlying mechanisms responsible for SpA pathogenesis. Therefore, the following hypotheses have been constructed:

1) Anti-HSP60 antibodies are linked to disease activity in SpA. The aim of this study was to investigate if a relationship between antibodies to the immunogenic heat shock protein 60 (HSP60) and clinical characteristics exist. Recombinant HSP60 was purified for human and 3 different bacteria known to trigger SpA disease. Furthermore, an enzyme-linked immunsorbent assay (ELISA) was established to determine levels of anti-HSP60 IgG subclass antibodies.

2) IgG subclass determination of the Anti-HSP60 response in SpA does not support cross-reactivity. The aim of the second study was to make a cohort study in which antibodies were determined over time. Furthermore, The IgG subclass specificity for bacterial and human HSP60 was to be investigated in more detail.

3) SNP's in the *IL-1A* gene affect kinetics in the IL-1α response. The aim of the third study was to investigate the IL-1α kinetics among human monocytes and macrophages. Secondly to develop a functional assay to investigate the importance of SNP's in *IL-1A* for use in a clinical study in which PBMC's from SpA patient collected in study I would serve as donors.

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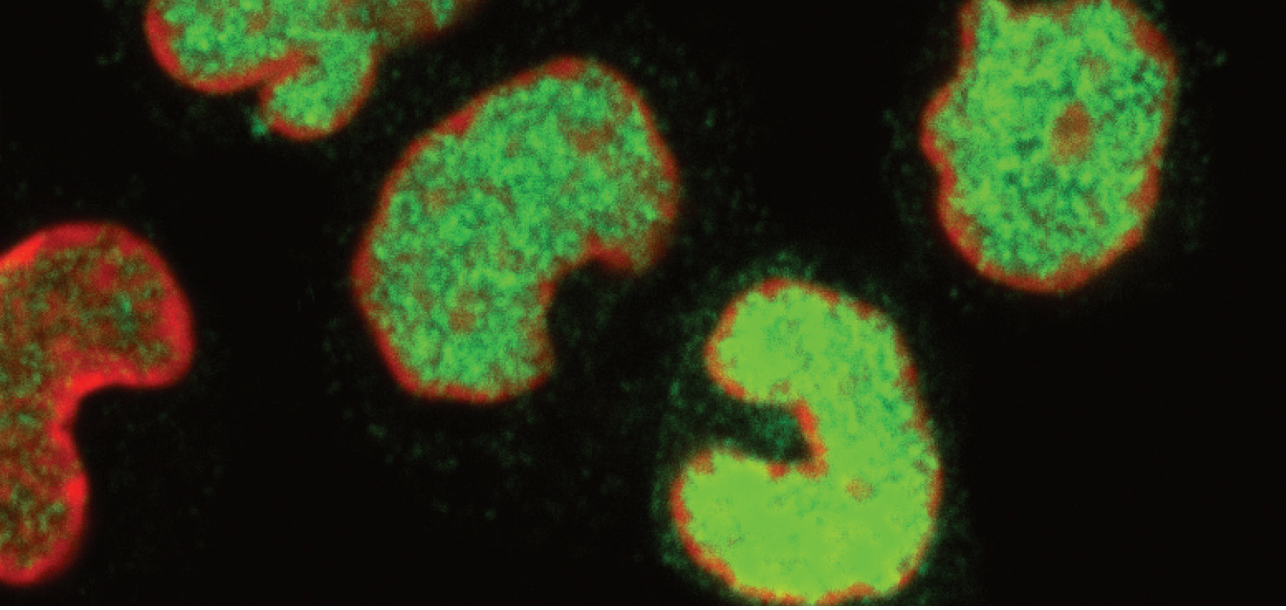

SUMMARY

This dissertation includes a presentation of knowledge on the molecular pathogenesis of spondyloarthritis achieved through a PhD programme at Aalborg University from 1.12.2011 - 1.12.2014.

Work was carried out in the Laboratory of Medical Mass Spectrometry, headed by:
Professor Svend Birkelund
Associate Professor Allan Stensballe

The output of this PhD programme, besides from this dissertation, includes 5 published papers, 30 ECTS PhD courses, oral presentations of posters in national and international research environment and a short-term scholarship at the La Jolla Institute for Allergy and Immunology in San Diego, USA.

Research was conducted on biological material from patients suffering from spondyloarthritis and healthy donors, to whom I dedicate this work.

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